LONG-TERM CHOLINERGIC DENERVATION CAUSED BY EARLY POSTNATAL AF64A LESION PREVENTS DEVELOPMENT OF MUSCARINIC RECEPTORS IN RAT HIPPOCAMPUS

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Long-term Cholinergic Denervation caused by Early Postnatal AF64A Lesion Prevents Development of Muscarinic Receptors in Rat Hippocampus

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ABSTRACT

The effect of early postnatal (day 8) intracerebroventricular injections of the putative cholinotoxin ethylcholine aziridinium mustard (AF64A) on development of cholinergic innervation and postsynaptic muscarinic acetylcholine receptors in the rat hippocampus was examined. The cholinotoxin applied at this stage of development leads to a permanent denervation of cholinergic fibres in the hippocampus in adulthood demonstrated by (immuno)histochemical methods and biochemical assays. Muscarinic receptor expression in the principal neurons of dentate gyrus and cornu ammonis was strongly reduced as studied by immunostaining with antibodies against muscarinic receptor proteins and binding assays with the muscarinic antagonist quinuclidinyl benzilate. Cholinoceptive interneurons and somatostatinergic interneurons are not affected by the developmental cholinergic lesion. Immunoreactivity to protein kinase C type I as a marker for inositolphosphate-related cellular activation systems slightly decreased in the apical dendrites of the hippocampal principal neurons. These findings indicate that damage to ingrowing cholinergic terminals in the hippocampus in the early postnatal period is a critical hazard for development of the muscarinic receptor system in the hippocampal principal neurons. These results are discussed for their significance to the neural mechanisms that underlie perinatal brain damage and associated cognitive dysfunction.

KEY WORDS: Perinatal damage Cholinergic innervation

INTRODUCTION

In recent papers we reported the effects of pre- and early postnatal hypoxia and anoxia in the rat on the development of serotonergic and cholinergic system development in cortex and hippocampus (Nyakas *et al.*, 1989). Furthermore, the effects of the perinatal hypoxic treatment were assessed on development of cognitive and motivational behaviours in which the above-mentioned forebrain transmitter systems and their receptors play a prominent role (Deutch, 1971; Whishaw *et al.*, 1985; Altman *et al.*, 1987; Nakahara *et al.*, 1989; Nyakas *et al.*, 1990, 1991). It was demonstrated that hypoxic events in the perinatal period yield a suppression of ingrowth of cholinergic and serotonergic fibres in cortex and hippocampus during their development in the first 2 weeks after birth. Littermates receiving the same treatment revealed a severe malfunction of open-field motor behaviours and of learning and memory performance (Nyakas *et al.*, 1989, 1990, 1991).

This coincidence of neuropathologic damage to hippocampal and cortical systems and hippocampal behavioural functions after hypoxia suggests causal relationships between these affected systems and abnormal behavioural function. Hypoxia and anoxia, however, exert a rather general effect on the developing nervous system, although some areas like cortex and hippocampus are more susceptible to hypoxia than others (Siesjö and Plum, 1973; Fenichel, 1980; Onodera *et al.*, 1987; Mudrick and Baimbridge, 1989; Represa *et al.*, 1989; Choi, 1990; Vannucci, 1990). Furthermore, hypoxia and ischaemia have a reported depressive effect on synthesis of cholinergic enzymes and influence receptor density in the developing brain (Gibson and Duffy, 1981; Gross *et al.*, 1981; Hershkowitz *et al.*, 1983; Speiser *et al.*, 1986).

The present experiments were undertaken to gain further insight in the effect of specific cholinergic lesions in the perinatal period on the development of the muscarinic acetylcholine receptor (mAChR).

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This development of mAChR has our particular interest since it plays a key role in learning processes and other cognitive functions (Deutch, 1971; Whishaw *et al.*, 1985; Mudrick and Baimbridge, 1989). To this aim we investigated developmental effects of specific cholinergic lesions in the hippocampus in the early postnatal period. The lesions were obtained by intracerebroventricular (i.c.v.) injections of the cholinotoxin ethylcholine aziridinium mustard (AF64A), which at low doses and in certain experimental conditions is a selective toxin to hippocampal cholinergic innervation (Fisher *et al.*, 1982; Kása *et al.*, 1986; Potter *et al.*, 1986). With such a tool we aimed to assess the effects of specific damage to growing cholinergic fibres and development of mAChR receptors in the hippocampus. In this paper we will report on the long-term effect of early postnatal lesion on development of (1) hippocampal cholinergic innervation, (2) postsynaptic muscarinic receptors, (3) protein kinase C (PKC) immunoreactivity, and (4) somatostatinergic innervation of the hippocampus. PKC was studied because of the intimate functional relations of this enzyme with muscarinic receptors (Haga *et al.*, 1990; Strosberg, 1991). Somatostatin (SOM) immunostaining was investigated in view of the close anatomical contacts between septo-hippocampal projections and SOM-positive target interneurons in the hippocampus (Yamano and Luiten, 1989) and the recently demonstrated colocalization of mAChR and SOM in such cells (Van der Zee *et al.*, 1991). Effects of perinatal AF64A lesion on development of cognitive behavioural performance will be described in a separate report. Part of the current data were recently presented in abstract form elsewhere (Luiten *et al.*, 1990).

MATERIALS AND METHODS

Lesioning procedure with AF64A

For the present investigation, 37 male Wistar pups were injected with the cholinotoxin AF64A (RBI, Natick, USA) or the vehicle solution. Before injection, the acetyl ethylcholine mustard was subjected to basic hydrolysis and subsequent transition into an aziridinium ion as described by Potter *et al.* (1986). At postnatal day 8, randomly selected pups from various nests were lightly but adequately anaesthetized with ether for approximately 10 min. The drug was injected bilaterally over a period of 5 min per injection for each side of the brain. The deposits were carried out with a 5 µl Hamilton syringe adjusted to the micromanipulator of a stereotaxic instrument. The tip of the injection needle was aimed at the lateral ventricles at stereotactic coordinates relative to bregma AP 0.0, L ± 1.4 and V 4.0. The ventral coordinate was measured from the surface of the skull. AF64A was delivered at an optimal dose of 2.0 nmol in 2 µl buffered saline per injection (Gower *et al.*, 1989). Control animals (shams) were injected in a similar way but only received 2 µl of the vehicle solution per injection.

After injections the pups were randomly distributed among the mother rats so that each mother nursed seven to eight pups. All experiments were terminated when the animals reached the age of 3 months.

Tissue preparation

To study the effect of postnatal administration of AF64A on the development of the cholinergic innervation in the forebrain, the cholinergic fibres were stained by use of acetylcholinesterase (AChE) histochemistry and in most of the cases also by immunocytochemistry for choline acetyltransferase (ChAT). For all (immuno)histochemical treatments, the animals were transcardially perfused with 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% saturated picric acid in 0.1 M-phosphate buffer (PB) at pH 7.4. The brains were dissected, stored overnight in 30% sucrose at 4°C and serially sectioned on a cryostat microtome at thicknesses of 16 and 30 µm.

For AChE histochemistry, free-floating sections were postfixed for 12–24 h in 2.5% glutaraldehyde in 0.05 M-PB and stained according to the procedure as described by Hedreen *et al.* (1985). In short, this procedure included reactions in subsequent steps of acetylthiocholine, ammonium sulfide, sodium nitrate and silver nitrate, yielding a clear staining pattern of AChE-positive fibres.

Immunocytochemical staining for ChAT and SOM started with pretreatment with 10% normal rabbit or normal goat serum, directly followed by exposure to goat anti-ChAT (Bruce *et al.*, 1985) or rabbit anti-SOM28 (Benoit *et al.*, 1982), respectively. The anti-ChAT antibody was kindly donated by Dr L. B. Hersh, the anti-SOM antibody was a gift of Dr R. E. Benoit. Then sections were incubated with rabbit anti-goat IgG or goat anti-rabbit IgG, followed by goat or rabbit peroxidase–antiperoxidase, respectively. All antibodies were diluted in phosphate-buffered saline (PBS) with 0.1% Triton X-100 (for further details on the procedures see Luiten *et al.*, 1988, and Van der Zee *et al.*, 1991). Protein kinase C subtype I was immunostained by using monoclonal antibody 36G9 against the purified enzyme (Cazaubon *et al.*, 1989). Incubation steps included 5% normal sheep serum, mouse IgG anti-PKC-I (1:200, overnight at 4°C), biotinylated sheep anti-mouse IgG (Amersham, 1:200, 2 h at room temperature) and horseradish peroxidase (HRP)-conjugated streptavidin (Amersham, 1:200, 2 h at room temperature). The immunocomplexes were visualized by standard reaction with diaminobenzidine (DAB) and hydrogen peroxide.

Immunocytochemistry for mAChR

Muscarinic receptors were visualized with monoclonal antibody M35 raised against purified receptor

proteins (see André *et al.*, 1987; Luiten *et al.*, 1988; Van der Zee *et al.*, 1989; Schröder *et al.*, 1990, for descriptions of procedures, characterization and applications). For mAChR immunocytochemistry, animals were perfused with 300 ml fixative containing 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M-PB. The brains were dehydrated overnight in 30% sucrose solution and cut to 30 μm frozen sections. The sections were pretreated with 0.1% H_2O_2 and 5% normal rabbit serum, immediately followed by incubation with M35 (mouse IgM) overnight at 4°C. After rinsing, the sections were exposed to biotinylated rabbit anti-mouse IgM for 2 h at room temperature and subsequently to streptavidin conjugated with HRP, again for 2 h at room temperature. All incubations took place in PBS but without any addition of detergents. The HRP labels were finally stained with DAB and H_2O_2 in Tris buffer.

Neurochemical determinations

To avoid blood contamination of brain tissue, the rats were transcardially perfused with heparinized saline under pentobarbital anaesthesia. The brain was rapidly removed from the skull and several brain areas were dissected out macroscopically: hippocampus, parietal neocortex and corpus striatum; the activity of the two cholinergic marker enzymes ChAT and AChE was determined. For ChAT measurement, the method described by Brownstein *et al.* (1975) was followed. Briefly, the tissue samples were homogenized in 0.1 M-PB, pH 6.0. The final incubation volume of 40 μl contained 0.25 M-NaCl, 0.1 mM-neostigmine, 12.5 mM-choline chloride, 0.1% Triton X-100 and 1.25 mM-[^{14}C]acetyl coenzyme A (5 mCi/mmol, Amersham). After incubation at 37°C for 30 min, the samples were passed through DOWEX 1 (HCO_3^-, Serva, Heidelberg, FRG) for elimination of labelled acetyl coenzyme A. The activity of the enzyme was expressed in μmol acetylcholine synthesized per hour per 1 g protein of the sample.

The activity of AChE was assayed with the colorimetric method of Ellman *et al.* (1961). The tissue samples were homogenized in 50 vol. 0.1 M PB, pH 8.0, containing 1% Triton X-100. The enzyme activity was expressed in μmol/min per g protein. For protein measurement, the method of Lowry *et al.* (1951) was used.

Biochemical determination of muscarinic binding sites

The number of muscarine-like binding sites was measured by a modified method of Yamamura and Snyder (1974). Briefly, the brain tissue was homogenized in 20 vol. ice-cold sucrose (0.32 M) and centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged again at 17,000 *g* for 15 min and the pellet resuspended and homogenized in 0.32 M-sucrose. Aliquots of this homogenate (protein contents varied between 0.15 and 0.20 mg) were incubated in the presence of a saturating concentration (2 nM) of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB; 42 Ci/mmol, Amersham, UK) in a 50 mM-sodium-phosphate buffer, pH = 7.4 at 25°C for 60 min. After incubation, the samples were filtered on Whatman GF/B glass microfibre papers, which were washed twice with 10 ml ice-cold PB. The bound radioactivity retained on the filters was counted with a liquid scintillation spectrometer and corrected for non-specific binding, which was measured in the presence of 10^{-4} M-atropine. The protein content was quantified according to Lowry *et al.* (1951).

RESULTS

All cases that received bilateral intraventricular injections with the cholinotoxin AF64A at postnatal day 8 revealed a very strong and consistent reduction of cholinergic fibres in the hippocampus (Fig. 1). This reduction was visualized both by AChE and by ChAT staining of fibres and affected all areas from cornu ammonis (CA) and dentate gyrus (DG). It appeared, however, that the cholinergic denervation in the DG was slightly stronger than in the CA. This may be explained by a relatively larger proportion of axons traversing the stratum oriens (Or) being less sensitive to AF64A than the larger proportion of terminals in the dentate at the time of the lesion. The general reduction of cholinergic fibres in the hippocampus only showed a limited variation. In the majority of cases, the neurotoxin treatment resulted in a fibre decrement indicated as 'moderate'. In some cases, there was an almost total depletion of cholinergic fibres, which was notably the case in the DG. In the 'moderate' lesion cases, some non-specific tissue necrosis was observed near the site of the needle track. This non-specific damage was accompanied by a slight reduction in size of the fimbria-fornix and an enlargement of the dorsal part of the lateral ventricle. In some more heavily lesioned cases also, some damage to the CA_3 area of the dorsal hippocampus occurred close to the injection needle track. Such damage, however, did not involve the CA_1 region. Furthermore, as a general observation it was noted that the lesion effects on cholinergic innervation development were stronger in the dorsal hippocampus as compared to the ventral or temporal hippocampal areas. This difference most likely is to be related to the dorsal and anterior position of the neurotoxin injection in the ventricle. In contrast to the consistently large decrease in cholinergic fibre density in the hippocampus, there were no such effects observed in the neocortex nor in the striatum.

Similar results were obtained when assessing cholinergic activity by measuring the ACh synthesizing and degrading enzymes by means of

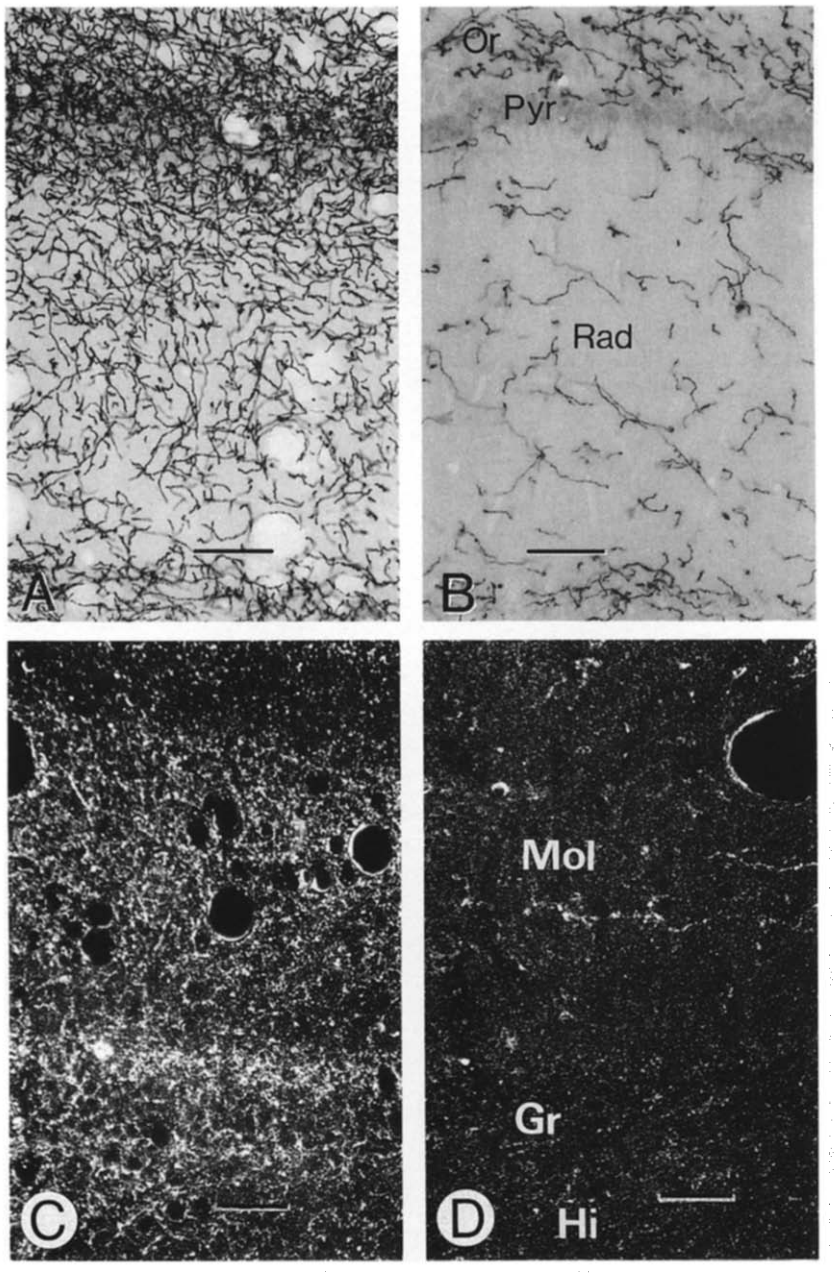

Fig. 1. Photomicrographs showing the effects of early postnatal intracerebroventricular injections of AF64A on cholinergic innervation of the hippocampus in adulthood. (A) and (B) illustrate cholinergic fibres stained for acetylcholinesterase activity in the cornu ammonis, in controls (A) and in lesioned cases (B). Lesion effects exemplified for the dentate gyrus are indicated in (C) and (D) by dark-field illumination of fibres stained for choline acetyltransferase immunoreactivity in controls (C) and AF64A-lesioned cases (D). Scale bar in all figures = 50 μm.

biochemical assays in tissue homogenates. Both enzymatic markers revealed a highly significant decrement of approximately 60% in adulthood in the hippocampi of AF64A-lesioned animals (Fig. 2) but not in the neocortex and striatum.

To establish the effect of an early postnatal and persistent cholinergic lesion in the hippocampus on development of postsynaptic cholinergic receptors, the presence of muscarinic receptors was investigated by binding of ligands and antibodies to mAChR. Immunoreactivity of anti-muscarinic monoclonal antibody M35 displayed a strikingly lower level of antibody binding in the AF64A-treated cases in adulthood. Similar to the effects found on cholinergic fibre markers, both CA and DG reacted with equally low levels of immunostaining, both of the cell bodies in the granular (Gr) and pyramidal (Pyr) cell layers, and in the DG molecular (mol) and CA dendritic layers. On the other hand, there was no decrease in immunostaining in interneurons situated close to the pyramidal cell layer or in the dentate hilar region. It

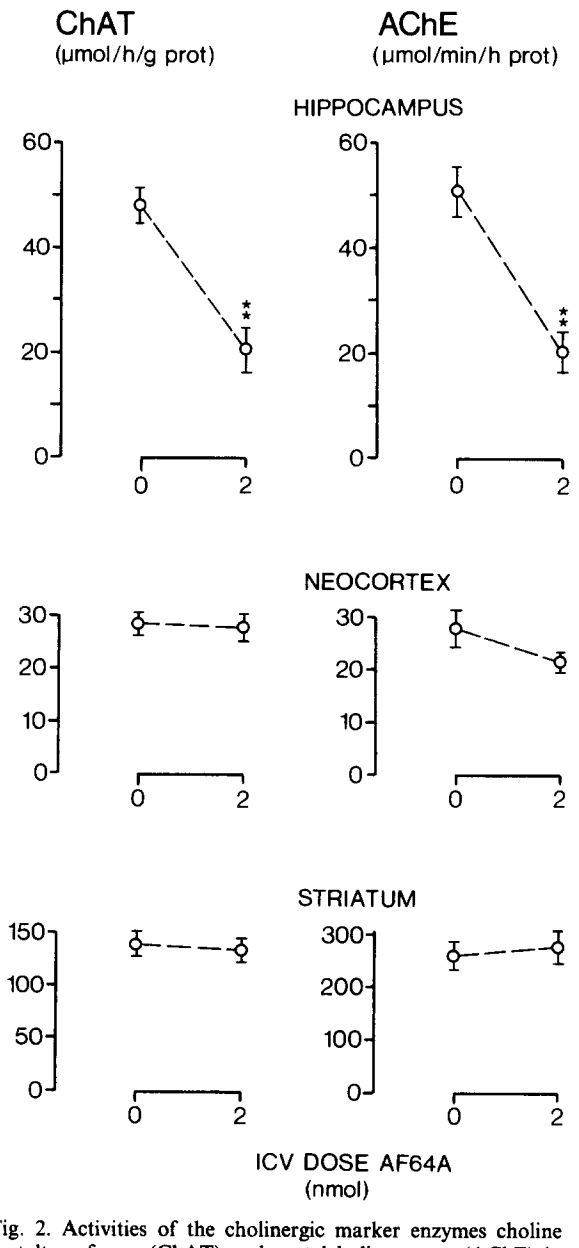

Fig. 2. Activities of the cholinergic marker enzymes choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in hippocampus, parietal cortex and striatum in controls and rats postnatally injected with 2 nmol AF64A in the lateral ventricles. In the hippocampus both enzyme activities are significantly decreased after AF64A treatment (**$P<0.001$).

even appeared that those cells in the AF64 cases stained somewhat stronger (Figs 3, 4). Again, the lesion effects were confined to the hippocampus.

Identical effects of the perinatal lesion on muscarinic receptors were established by binding of the tritiated muscarinic ligand [^{3}H]QNB in hippocampal tissue homogenates. A consistently and significantly lower level of 60% to controls was measured in the lesioned hippocampi (Fig. 5).

In spite of the dramatic and persistent depletion of cholinergic innervation of CA and DG, there was no obvious change in the long-term development of the SOM-immunoreactive interneurons of the hippocampus. These SOM interneurons characteristically made up a part of the interneuron population in the hilar (Hi) region of the DG and the stratum oriens (Or) of the CA. Notwithstanding the apparent cholinergic input to these neurons, there was no clear change in cell number nor in the SOM projections to the molecular layers of the hippocampus. In four control cases and five representative AF64A lesioned cases, the numbers of SOM-immunoreactive cells were quantified in the dorsal hippocampus at AP level 5.20–5.40 mm anterior to interaural. SOM-immunoreactive neurons were counted in three transverse sections per case and calculated as average numbers of cells per mm^2 tissue in dentate hilus and CA_1 stratum oriens. Oriens and hilus in controls contained 98.4 ($\pm$15.1) and 73.1 ($\pm$2.4) cells/mm^2, respectively, while these regions in the AF64A-treated animals contained 101.8 ($\pm$13.8) and 66.4 ($\pm$3.7) cells/mm^2. This means an increase of 3% SOM cells in the oriens and a decrease of 9% in the hilus in the AF64A cases relative to controls. These differences are not significant. Similar observations were made on PKC-I immunoreactivity of the hippocampus. PKC type I, which for a considerable part is colocalized with M35, revealed patterns that were only slightly influenced by the AF64A treatment. The PKC-I activity was notably strong in the pyramidal cells of the CA and their apical dendrites, whereas the activity level is only minor in the DG granule cells. This pattern was similar in the AF64 cases, although a slightly less intense staining in the dendritic region of the stratum radiatum was observed.

DISCUSSION

The present data show that destruction of ingrowing cholinergic fibres in the hippocampus in the early postnatal period results in a permanent cholinergic denervation. This cholinergic depletion coincides with a suppressed muscarinic acetylcholine receptor expression in the postsynaptic cholinoceptive principal neurons of the hippocampus, whereas this was not the case in some cholinoceptive interneurons. The cholinergic breakdown after intracerebroventricular injection with the cholinotoxin AF64A is a phenomenon well-documented with anatomical, histochemical and biochemical data (Gaál *et al.*, 1986; Kása *et al.*, 1986; Gower *et al.*, 1989). AF64A acts at the high-affinity choline uptake mechanism at cholinergic nerve endings (Chrobak *et al.*, 1989). It was shown by various authors that i.c.v. injections of low doses of 5 nmol AF64A in the lateral ventricle selectively destroy presynaptic cholinergic terminals (Gaál *et al.*, 1986; Kása *et al.*, 1986; Gower *et al.*, 1989) and by retrograde transport via axonal pathways affect the cell bodies of origin in the medial septum complex (Gaál *et al.*, 1986). The specificity of AF64A as a cholinotoxin, however, is still a matter of debate. AF64A injected directly into

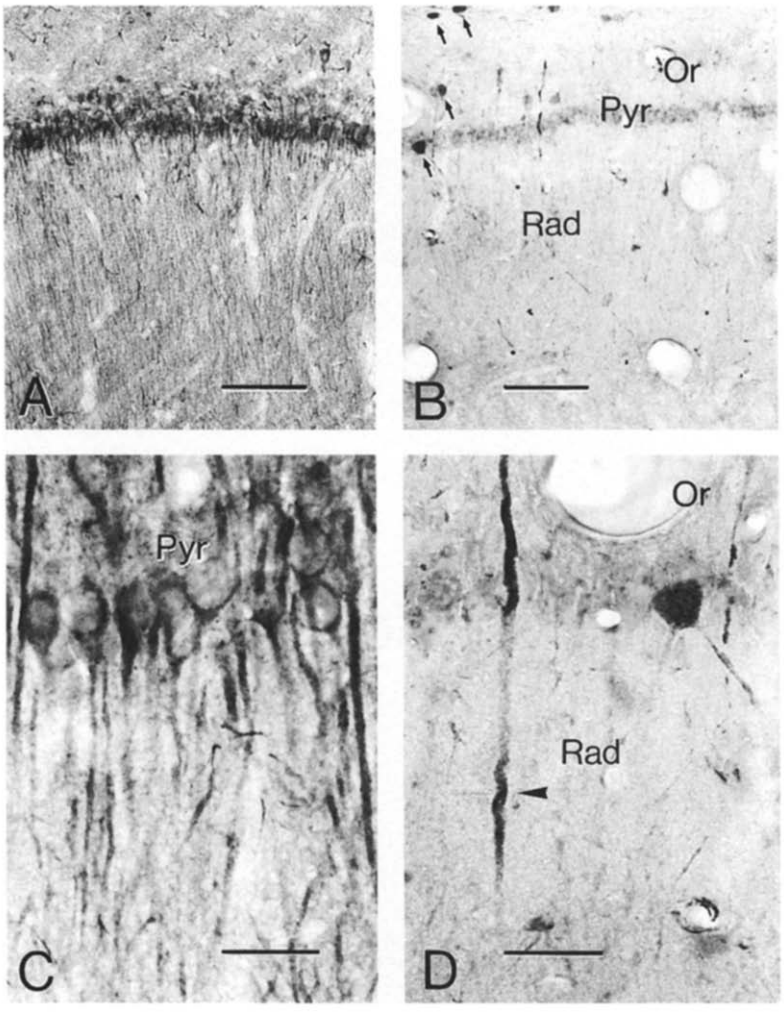

Fig. 3. Immunostaining to muscarinic receptors in the cornu ammonis$_1$ at low (A,B) and higher magnifications (C,D) in controls (A,C) and after AF64A lesion (B,D). Note the decreased immunostaining in the pyramidal cells in the lesioned cases, whereas interneuron staining (arrows) remains unchanged. Scale in A,B: 100 μm; in C,D: 25 μm.

brain tissue not only acts as a cholinotoxin but causes damage to identified non-cholinergic systems as well, even when low doses of 0.05 nmol are applied (Levy *et al.*, 1984; Kozlowski and Arbogast, 1986; McGurk *et al.*, 1987). Higher AF64A concentrations of 3 nmol or more, when injected into the lateral ventricles, also may induce damage to non-cholinergic systems and result in tissue necrosis (Jarrard *et al.*, 1984; Gaál *et al.*, 1986; Potter *et al.*, 1986; Hörtnagl *et al.*, 1987). As was also the case in our own experiments, AF64A close to the site of injection resulted in local tissue damage and ventricle enlargement that cannot be attributed to a specific anti-cholinergic action of this neurotoxin. However, when applied in relatively low doses of 2 nmol or less, combined with administration via the ventricular system (Kása *et al.*, 1986), the non-specific damage appears to be limited to the direct vicinity of the injection locus. As such, observations on the anti-cholinergic effects of the AF64A injections should be carried out on brain regions at a safe distance from the injection site where no apparent necrosis can be detected. Furthermore, it was previously demonstrated that the hippocampus reveals the highest sensitivity to AF64A (Fisher *et al.*, 1982; Gower *et al.*, 1989). In the present study as well, the damage after low-dose i.c.v. injections of 2 nmol AF64A remains confined to the hippocampus and has no or only minor effects on other forebrain areas (Fisher *et al.*, 1982; Kása *et al.*, 1986; Gower *et al.*, 1989).

Apart from the toxic effect of AF64A on presynaptic structures, several investigators reported lack of changes in postsynaptic acetylcholine receptors as studied by ligand binding techniques (Fisher *et al.*, 1982; Kása *et al.*, 1986). The absence of effects of AF64A treatment on cholinergic receptor affinity or expression in their experiments, however, is not very surprising. Similar lack of effects on muscarinic receptors is reported after fimbria-fornix and medial septum lesions (Sábato *et al.*, 1981) and in pathological cholinergic breakdown as in Alzheimer's disease (Giacobini *et al.*, 1988; Geula and Mesulam, 1989; Luiten *et al.*, 1989; Schröder *et al.*, 1991).

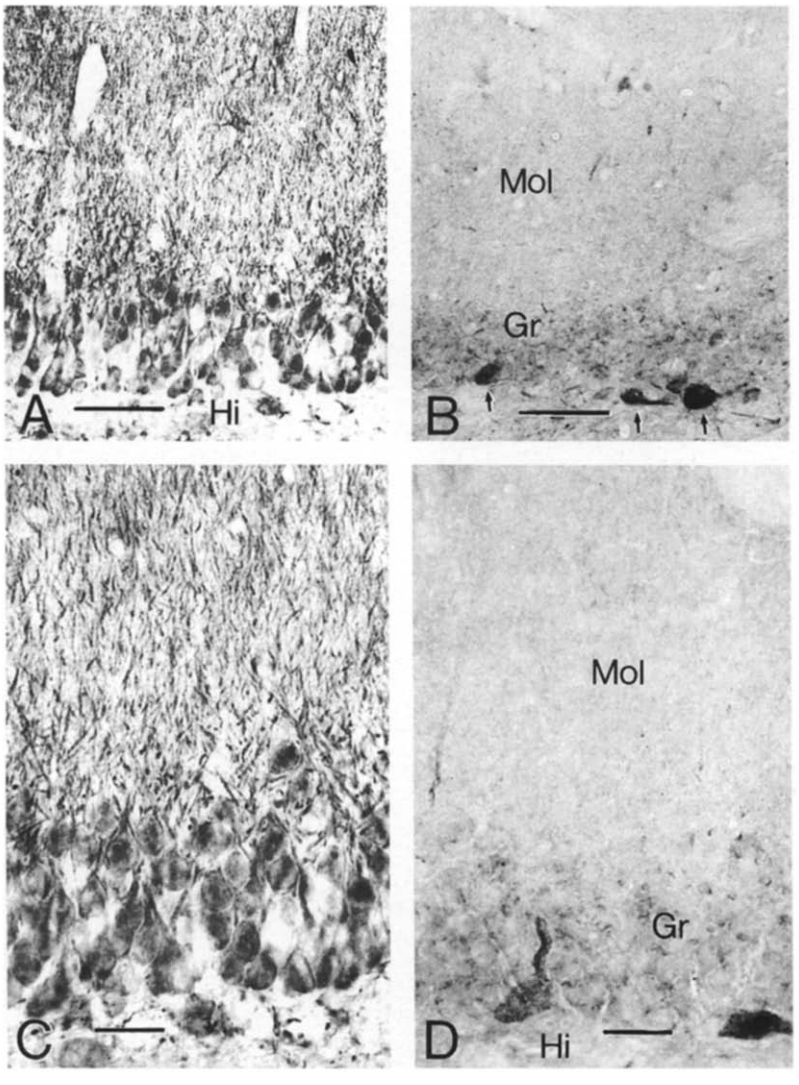

Fig. 4. Immunoreactivity to muscarinic acetylcholine receptor with antibody M35 in the dentate gyrus granular and molecular layers in controls (A,C) and AF64A-lesioned rats (B,D). As in the cornu ammonis$_1$ region, decreased staining is limited to the granular cells, while the interneurons stain as normal. Bar in A,B: 50 μm; in C,D: 25 μm.

The difference with the currently used experimental set-up, however, is the fact that in our case mild (2 nmol) i.c.v. injections of AF64A were applied at early postnatal periods. In this period the cholinotoxin interferes with the process of ingrowth of cholinergic fibres from their subcortical sources in medial septum complex to their hippocampal target cells (Milner *et al.*, 1983; Nyakas *et al.*, 1987, 1989). At the time of lesion at postnatal day 8, the cholinergic fibres start to invade the hippocampal formation and the process of synaptogenesis is only in its primary stages (Kolb and Whishaw, 1989; Mattson, 1989). So the lesion coincides with the period when most synapses have not yet formed or when stabilization of functional contacts has not yet occurred (Berry, 1974; Kolb and Whishaw, 1989; Vaughn, 1989). Maturation of the cholinergic septo-hippocampal connection continues even up to 50 days after birth (Sofroniew *et al.*, 1987).

In the early period of postnatal development, there is a gradually increasing expression of muscarinic receptors, which has been studied by muscarinic ligand binding and by gene expression (Coyle and Yamamura, 1976; Rotter *et al.*, 1979; Large *et al.*, 1985; Fiedler *et al.*, 1987; Noguchi *et al.*, 1988; Pinkas-Kramarski *et al.*, 1989). The ontogeny of muscarinic receptors parallels the development of secondary messenger systems that are linked to mAChRs (Strosberg, 1991), like phosphatidylinositol turnover (Large *et al.*, 1985; Balduini *et al.*, 1987) and PKC activity (Nakahara *et al.*, 1989; Sposi *et al.*, 1989). It was also demonstrated that muscarinic receptors are functionally coupled to their second messenger systems, before functional synaptic contacts are established (Large *et al.*, 1985; Balduini *et al.*, 1987). Since the current experiment is characterized by cholinergic fibre destruction by AF64A before or during synapse formation, the

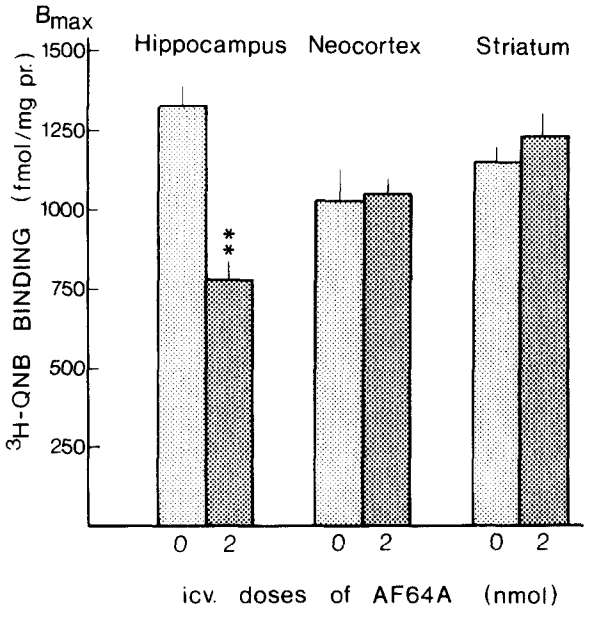

Fig. 5. Histograms showing levels of binding of the labelled muscarinic antagonist quinuclidinyl benzilate (QNB) in hippocampus, cortex and striatum at the age of 3 months. The total labelling (B_{max}) in the hippocampus is significantly reduced (**$P<0.01$) in the perinatally AF64A-lesioned animals.

effects on muscarinic receptor development and expression are probably explained by absence of presynaptic trophic influence on postsynaptic receptor stabilization. This implies that the timely arrival of the presynaptic bouton is extremely essential for further development and maturation of the muscarinic receptor complex (Nyakas *et al.*, 1989; Represa *et al.*, 1989), which contrasts with the lack of such presynaptic influence on postsynaptic muscarinic receptor expression and function in adulthood and aging. In other words, these findings indicate that presynaptic cholinergic input is essential for the formation of synaptic integrity, but is not relevant for the maintenance of muscarinic receptor function after synaptogenesis. The latter phenomenon is also illustrated by recovery of functional deficits by application of muscarinic agonists in AF64A-lesioned animals (Nakahara *et al.*, 1989).

In the current study it was also established that the persistent cholinergic denervation had no or only minor effects on the immunoreactivity to PKC-I. This was somewhat surprising since the M1 subtype of the muscarinic receptor is intimately linked to phosphatidylinositide turnover and PKC activation (Haga *et al.*, 1990; Strosberg, 1991). This coupling of mAChR and PKC-I was recently also demonstrated by colocalization studies with double-label immunocytochemistry (Van der Zee *et al.*, 1990). On the other hand, it has been reported that in early ontogeny only the II- and III-subtypes of PKC are expressed, whereas the gene of PKC-I becomes activated much later in development (Sposi *et al.*, 1989). Furthermore, we should realize the PKCs are associated not only with cholinergic input, but also with a large number of receptors for other input sources to hippocampal principal cells, such as glutamate, serotonin, norepinephrine and a variety of neuropeptides and hormones (Strosberg, 1991). It was also striking that the permanent cholinergic breakdown apparently did not exert much influence on the mAChR immunoreactivity, nor on the transmitter content of hippocampal interneurons such as the SOM cell group. SOM cells in the hippocampus were shown to receive a partly cholinergic medial septal input and half of the SOM neurons are provided with muscarinic receptors (Yamano and Luiten, 1989; Van der Zee *et al.*, 1991). We do not known, however, whether the M35-immunoreactive interneurons surviving after cholinergic lesion in the present study are also SOMergic cells. The fact that SOMergic neurons remain unaffected by the developmental cholinergic lesion indicates that its cholinergic afference is not essential for its neuronal development in adulthood. The observation that SOM immunoreactivity does not change after chronic cholinergic denervation induced by AF64A treatment corroborates previous findings that SOM-immunoreactivity in the hippocampus remained unaltered after septo-hippocampal lesions in adult hood (Pierotti and Simpson, 1986). The condition of SOM after AF64A lesions, however, is dependent on the post-lesion survival time. Hörtnagl *et al.* (1990) recently reported that SOM concentrations in the hippocampus show a transient reduction after cholinergic denervation by AF64A, and recover to control levels 2 weeks after lesion. The relatively strong M35 immunoreaction in the interneurons shows that the suppressed maturation of mAChR in the principal cells is a rather cell-specific effect with yet unknown underlying mechanisms.

We should bear in mind, however, that AF64A lesions may also influence other transmitter or neuroactive systems in the developing hippocampus. We cannot entirely rule out that AF64A may have affected the development of monoaminergic systems in the hippocampus. In this respect it has been reported that higher concentrations of AF64A injections induce a transient reduction of serotonin levels (Potter *et al.*, 1986), which may play a role in the maturation of hippocampal tissue (Hamon *et al.*, 1989).

A further question we wish to answer is the assumed relationship between growth-retarded cholinergic development after perinatal hypoxia, and the causal mechanisms of learning defects after such hypoxic events. The current experiments, albeit with an exaggerated perinatal cholinergic lesion, clearly indicate the detrimental effects of the cholinergic lesion on muscarinic receptor expression. It may be concluded also that less dramatic suppression of cholinergic hippocampal innervation will probably have a suppressive influence on mAChR development (see also Represa *et al.*, 1989). Such a muscarinic receptor suppression will likely add to the persistent cognitive decline

observed after perinatal hypoxic brain damage (Hershkowitz *et al.*, 1983; Nyakas *et al.*, 1989, 1991).

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